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Cu^{2+} uptake by *Chlorococcum hemicolum* - A Xeric Chlorophycean Alga

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Abstract

Bioremediation of copper by xeric chlorophycean bioremediator, *Chlorococcum hemicolum* was investigated. The growth rates at various concentrations of Cu^{2+} were assessed in terms of protein level and 8 mg L^{-1} (37.67 % level in growth kinetics) is the tolerance limit. Absorption/adsorption kinetics was estimated after 240 hrs of Cu^{2+} treatments. Absorptions were higher than adsorption with maximum accumulation factor (AF) of 1.40. The Cu^{2+} concentration and absorption were linearly related ($r = 0.99$; $p>0.01$). Other biochemical parameters like total sugar, chlorophyll and carotenoids were also quantified to correlate the state of metabolism and these exhibited reduction due to heavy metal stress.

Keywords: Bioremediation, *Chlorococcum hemicolum*, Copper, Hyperaccumulator.

1. Introduction

Indian Thar Desert has been experiencing increased mining and industrial activities during the last few decades; a consequence of this is pollution, particularly water pollution, in scarce water resource of the Thar Desert. A case of anxiety is Boranada Industrial Area, Jodhpur, where the dye of fabrics and metal alloy productions are the major industrial activities, releasing effluent containing dangerous level of heavy metals, which flow into the local Jojri River. The release of copper and other metals into natural water systems as a result of industrial processes has led to increased concern about the effects of toxic metals in the environment (Long et al., 2009; Lee and Chang, 2011). Copper is an essential trace element for plants, it participates in photosynthetic electron transport and plays cofactor role in several oxidizing enzymes. Trace amounts of copper are essential for metabolic processes of algae (Li et al., 2006); it has particular significance for plastocyanin. Higher concentrations are toxic and for many years copper sulphate has been widely used as an algicide to control or prevent undesirable algal growth, particularly, waterblooms. Copper toxicity results in an inhibition of electron transport (Mohanty et al., 1989) and inhibition of

photosynthetic pigments (Fathi et al., 2000). The ability of micro-algae to remove heavy metals from aqueous solution has been known for some decades. Solisio et al. (2006) demonstrated the Cu^{2+} uptake capacity of *Spirulina platensis*. Biosorption of Cu^{2+} using marine algae *Gelidium* has also been investigated (Vilar et al., 2008). Recently, molecular characterization of copper resistant bacterium was also reported (Fan et al., 2011). The algae of chronically metal-contaminated localities tend to accumulate heavy metals to a dangerous extent. The metal content of algae can be used to predict the level of metal pollution in a water body. The high accumulation capacity can even be used for the enrichment or recycling of valuable metals (Harish et al., 2009). Their relative comparison is generally made with the help of accumulation factor (AF). Isolation of algal ecotype that can tolerate elevated metal concentrations, from local stream containing polluted industrial effluent, is essential for efficient functioning of bioremediation system in that particular environment. The study region (which comes under the area of Indian Thar Desert) is classified as semi arid. Consequently, the algal flora have considerable ecological importance, even if they occur in river micro-habitat (Harish et al., 2008). Hence, the aim of this study was to explore the biotic potential of local chlorophycean alga *Chlorococcum hemicolum* for Cu^{2+} bioremediation. Further changes in protein, sugar, chlorophyll and carotenoids were also assessed.

2. Materials and Methods

2.1. Experimental organism, growth and experimental conditions:

Chlorococcum hemicolum, a local alga, was collected from Jojri River (pH 6.2 – 6.5). This alga occurred at various locations within this stream. All the industrial effluent of nearby Boranada Industrial area, Jodhpur, flow into this stream. Axenic culture of this alga was multiplied in BG-11 medium (Rippka et al., 1979) and grown in culture room under continuous light, illuminated with cool fluorescent tubes (14.4 watt. m^{-2}) at 24±1°C.

All the experiments were conducted in triplicate at same culture conditions. The cultures contains glass beads (5 in number in each culture flask with 0.5 cm size each) to prevent clumping of cells in growing algal mass, and were shaken gently every day.

2.2. Changes in growth rate:

One mL of algal cells with protein value 100 µg mL^{-1} were withdrawn from exponentially growing homogenous culture of alga and inoculated in 100 mL freshly prepared BG-11 medium containing different ranges (0 to 14 mg L^{-1}) of Cu^{2+}. Source for Cu^{2+} in medium was stock solution prepared with $CuSO_4.5H_2O$. Changes in protein content were measured by the method of Lowry et al. (1951) as modified by Herbert et al. (1971) using lysozyme (Sigma) as the standard. Protein content was measured after every 48 hrs, up to 480 hrs, starting from 96 hrs (4^{th} day) from these newly established cultures.

2.3. Absorption/adsorption kinetics:

One mL of algal cells with protein value 100 µg mL^{-1} were withdrawn from exponentially growing homogenous culture of alga and inoculated in 100 mL freshly prepared BG-11 medium containing

different Cu^{2+} ranges (0, 2, 4, 6 and 8 mg L^{-1}). After 240 hrs of inoculation, 10 mL of algal sample was harvested from homogenous cultures and centrifuged (4000g, 15 minutes) and supernatant media were separated. The algal samples in the sediment were mixed individually with 10 mL of EDTA (10 µM) solution and gently shaken. Samples were once again centrifuged (4000g, 15 min.). Supernatant EDTA was taken out for measuring the adsorbed ionic concentration. All three parts i.e. media, EDTA and algal pellets from each sample were dried, digested with double acid [HNO_3: $HClO_4$ mixture (10:1, v/v)] in boiling water bath for 1 hr. After cooling, the samples were diluted to 10 mL with triple glass distilled water and analyzed for Cu^{2+} level by Atomic Absorption Spectrophotometer using Perkin Elmer model 373 AAS.

2.4. Other biochemical parameters:

Estimation of sugar: The soluble and insoluble sugars were estimated using the principle of hydroxymethyl furfural reaction with anthrone to produce green color (Plummer, 1971). Aqueous alcoholic soluble sugar part was considered as soluble and hydrolyzed part as insoluble (Plummer, 1971, Sadasivam and Manickam, 1992).

Estimation of chlorophyll and carotenoids: Pigments were extracted in methanol and their relative amounts were calculated using equation (Mackinney, 1941):

$13.42 \times A_{665}$ = µg chlorophyll mL^{-1}

$200 \times A_{420}$ = µg carotenoids mL^{-1}

Sugar and pigment were estimated 240 hrs after the different ranges of Cu^{2+} treatment.

All the experiments were triplicated and the results were statistically analyzed for variance (ANOVA) and cause effect relationship (Snedecor and Cochran, 1967). Growth rate experiment involved two factors (concentration of Cu^{2+} and period of growth) and performed as per strip-plot design, whereas in rest of the experiments concentration of Cu^{2+} is the only factor, accordingly they were carried out following randomized block design.

3. Results and Discussion

3.1. Changes in growth rate as measured by protein value:

The Cu^{2+} concentrations affected growth rate (Figure 1). The tolerance limit (sub-lethal concentration of Cu^{2+}) for *C. hemicolum* was observed to be 8 mg L^{-1} (> 50 % reduction in growth kinetics; 62.33%). The effect of sub-lethal concentrations of Cu^{2+} on cultures appears to delay the onset of exponential phase. Such marked increase in lag phase of the growth has been manifested earlier also (Anand et al., 2006; Harish et al., 2008). The results of factorial analysis of variance suggested that Cu^{2+} toxicity to growth rate is a consequence of Cu^{2+} dosage, duration of exposure and their strong interaction ($p>0.01$). Such results have been reported with cultures of *Scenedesmus* and *Ankistrodesmus* (Jin et al., 1996, Lin and Jiang, 2000). The results indicated that algal growth becomes oppressive as Cu^{2+} concentration increases, and ultimately inhibited at higher concentration (> 8 mg L^{-1}). Resistant cells, that survive the lag phase, are then able to enter the exponential phase of growth.

3.2. Absorption/adsorption kinetics:

Chlorococcum hemicolum is not only just a tolerant species but also found to be Cu^{2+} hyperaccumulator with maximum accumulation factor (AF = 1.40) found at 2 mg L^{-1} Cu^{2+} treatment (Table 1). Even at 4 mg L^{-1} Cu^{2+} treatment AF value was 1.38. AF curve shows parabolic path ($AF = 0.571 + 0.563X - 0.095 X^2$; $R^2 = 0.91$; X = Cu^{2+} concentration). The adsorptions were comparatively lower than absorption. However, both adsorption and absorption levels (Y) were linearly related with Cu^{2+} concentration [$Y = 0.068 + 0.091 X$; $r^2 = 0.74$ for adsorption; $Y = -1.89 + 2.081 X$; $r^2 = 0.99$ for absorption; Figure 2]. The variance analysis revealed that Cu^{2+} concentration contributed significantly to adsorption (F ratio 17.57; p>0.01) and absorption (F ratio 1740.43; p>0.01) levels. However, variations among the replicates were non-significant.

Bioremediation potential is judged on the basis of metal accumulation capacity per unit biomass at per unit time; hence, growth rate of biomass is an important factor. A low metal accumulator can out-compete a high metal accumulator on the account of high growth rate of biomass. In such cases, surface area to volume ratio also plays significant role. A win-win situation arises when high metal accumulator also have high growth rate with maximum surface area interface to metal in the medium. *Chlorococcum hemicolum* is able to meet these criterions being single cellular protista with high growth rate and high tolerance and high metal accumulation capacity (AF) to copper; this alga is a model for bioremediation systems in desert environment (Harish et al., 2008).

3.3. Other biochemical parameters:

The different range of Cu^{2+} treatments decreased both insoluble and soluble sugar and variations were significantly (F = 400.40; p>0.01 and 126.31; p>0.01, respectively) contributed by Cu^{2+} treatment only (Table 2). Insoluble sugar was found to be more than soluble sugar. Chlorophyll and carotenoid content reduced significantly with increase in Cu^{2+} concentration (F = 2036.4; p>0.01 and 8078.8; p>0.01, respectively; Table 2). Concentration dependent reduction of sugar and pigment was similar to those obtained earlier (Harish et al., 2008).

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Figure 1. Effect of Cu2+ on growth rate as measured by protein content in *Chlorococcum hemicolum*.

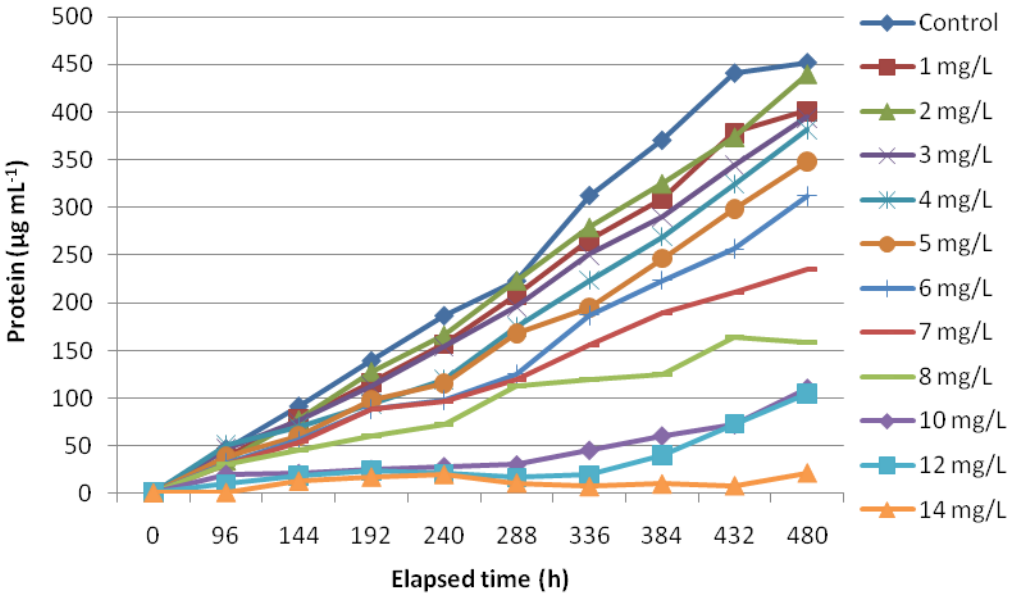

Figure 2. Copper absorption/adsorption kinetics of *Chlorococcum hemicolum*.

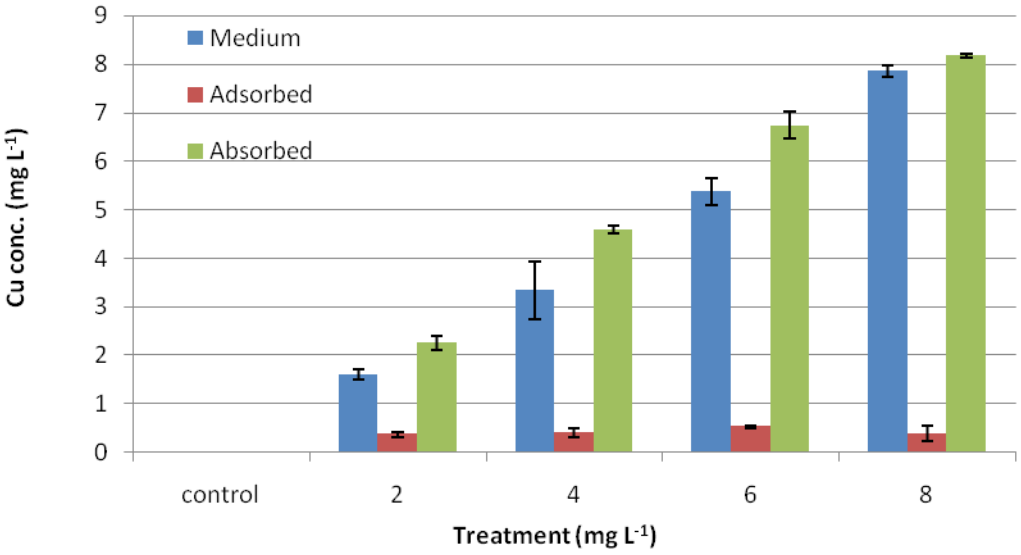

Table 1. Accumulation Factor for Cu^{2+} in *C. hemicolum*.

Cu^{2+} concentrations (mg L^{-1})	AF value
0	1
2	1.40
4	1.38
6	1.25
8	1.04

Table 2. Level of sugar and pigment in *Chlorococcum hemicolum* due to copper treatment

Cu^{2+} Concentrations (mg L^{-1})	Sugar (mg mL^{-1})		Pigment (µg mL^{-1})	
	Insoluble	Soluble	Chlorophyll	Carotenoids
0 (control)	0.069±0.001	0.018±0.002	11.134±0.252	172.333±1.286
2	0.040±0.002	0.011±0.001	4.612±0.122	76.867±1.137
4	0.020±0.003	0.009±0.001	1.548±0.156	39.667±0.503
6	0.008±0.002	0.005±0.001	1.203±0.063	25.867±1.222
8	0.001±0.002	0.002±0.0002	0.877±0.112	19.333±1.361
CD (at 0.05 level)	1.78E-02	7.14E-03	2.33E-02	1.14E-02